

Clinical Relevance of Matrix Metalloproteases and their Inhibitors in Breast Cancer

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Abstract

Degradation of the stromal connective tissue and basement membrane components are key elements in tumor invasion and metastasis. Some components, particularly the interstitial collagens, are very resistant to proteolytic attacks and can be degraded by specific proteinases like Matrix Metalloproteinases (MMPs). MMPs can also impact on tumor cell behavior *in vivo* as a consequence of their ability to cleave growth factors, cell surface receptors, cell adhesion molecules, or chemokines/cytokines, and for stimulating angiogenesis. Different molecular expression profiles of MMPs and their inhibitors (TIMPs) have been associated with the main steps in breast cancer progression, such as creating a potential invasive phenotype in Ductal Carcinoma *in situ* (DCIS), favoring the hematogenous dissemination, and enabling the metastatic progression across the axillary lymphatic system. These associations have clinical interests, as they can contribute to a better characterization of early breast carcinomas (which differ in their both biological and clinical behavior), evaluate microinvasion in resection specimens of breast tumors, provide a more precise prognostic, and predict the tumoral status of non-sentinel lymph nodes in breast cancer. It is also especially remarkable the evidences indicating that MMPs and TIMPs expression in individual cell populations from tumor stroma, such as mononuclear inflammatory cells (MICs) and fibroblast, clearly impacts on the clinical outcome of breast cancer patients. There are several factors linking inflammation, MMP activity and breast cancer. This knowledge will be useful to develop novel therapies and prevention strategies targeting critical components.

Keywords: Tumor invasion; Metastasis; Inflammation; Mononuclear inflammatory cell; Fibroblast

Introduction

Breast cancer is by far the most frequent neoplasm affecting women (23% of all cancers worldwide) [1]. Moreover, in spite of its increasing incidence, mortality has been rather stable for several years, being nowadays the first leading cause of cancer death [1]. This is due to the fact that although less than 10% of women with primary breast cancer have clinicopathological signs of disseminated disease at the time of the initial diagnosis, relapse in the form of metastases occurs in about half of the cases with originally apparently localized tumors within 5 years of surgery. However, it is difficult to predict the occurrence of distant metastases because breast cancer is a heterogeneous disease encompassing a variety of pathological entities and a wide range of clinical behaviors, even in patient groups that seem to be clinically similar. Therefore, and despite of having several classical prognostic variables available such as nodal status, tumor size, grade of malignancy, age and hormone receptor status, it is necessary to identify new prognostic factors in order to improve the current risk classification and thereby to develop a more rational management of breast cancer patients.

Tumor invasion and metastasis development are the primary determinants of patient outcome and, accordingly, molecules involved in these processes are obvious candidates to be identified as new prognostic markers in breast cancer. Different types of proteolytic enzymes (metallo-, aspartic-, cysteine-, serine-, and threonine-proteinases) perform the degradation of stromal connective tissue and basement membrane components, key elements in tumor invasion and metastasis [2]. However, some components, particularly the interstitial collagens, are very resistant to proteolytic attacks and can be degraded by specific collagenolytic enzymes like cathepsin K, neutrophil elastase and Matrix Metalloproteinases (MMPs) [3]. The human MMP family currently consists of 26 members of homologous zinc-dependent endopeptidases that can be divided into 6 structural classes

or, based on their substrate specificity and primary structure, [4-6] (Table 1). The expression of MMPs is induced by a variety of external stimuli such as cytokines and growth factors, including interferons, interleukins, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF- α) or beta (TNF- β), epidermal growth factor (EGF), and the extracellular matrix metalloproteinase inducer (EMMPRIN) [7]. MMPs are synthesized as inactive zymogens, which are then activated mainly pericellularly by either other MMPs or by serine-proteases. MMPs activity is specifically inhibited by the so-called tissue inhibitors of metalloproteases (TIMPs). Currently, 4 different TIMPs are known to exist: TIMP-1, 2, 3 and 4. These proteins perform the final regulation stage on the proteolytic activity of MMPs following the activation of the latent enzyme, and they are also endogenous inhibitors of members of a disintegrin and MMP (ADAM) family.

Collectively, MMPs are responsible for cleaving all of the major Extracellular Matrix (ECM) proteins and their balanced interaction with TIMPs regulates ECM homeostasis [8,9]. Balance of MMPs/TIMPs activity has a direct impact on mammary gland development and physiology, controlling mammary gland ECM remodeling during mammary morphogenesis, cyclical changes during the estrous cycle, and differentiation during lactation and mammary involution [10]. Hence, reduction of TIMP-1 expression through antisense RNA production

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MMP class	MMP	Enzyme name	Molecular Weight (kDa)	Substrates
Collagenases	MMP-1	Collagenase - I	57* 47 A	Collagens (I, II, III, VII, and X), proteoglycans, entactin, ovostatin, MMP-2, MMP-9.
	MMP-8	Collagenase-2/ neutrophil collagenase	85* 64 A	Collagens (I, II, III, VII, VIII and X), fibronectin, proteoglycans.
	MMP-13	Collagenase-3	60* 48 A	Collagens (I, II, III, VII, VIII and X), tenascin, plasminogen, aggrecan, fibronectin, osteonectin, MMP-9
	MMP-18	Collagenase-4	53* 51 A	Type I collagen
Gelatinases	MMP-2	Gelatinase-A	72* 66 A	Gelatin, collagen (IV, V, VII VI, IX and X), elastin, fibronectin.
	MMP-9	Gelatinase-A	92* 86 A	Collagens (IV, V, VII, X, and XIV), gelatin, entactin, elastin, fibronectin, osteonectin, plasminogen, proteoglycans.
Stromelysins	MMP-3	Stromelysin-I	60* 52 A	Collagens (IV, V, and IX), gelatin, aggrecan, laminin, elastin, casein, osteonectin, fibronectin, ovostatin, entactin, plasminogen.
	MMP-10	Stromelysin2	53* 47 A	Collagens (I, II, IV and V), gelatin, casein, elastin, fibronectin.
	MMP-11	Stromelysin2	60* 47 A	Collagens (IV, V, IX and X), laminin, elastin, fibronectin, casein, proteoglycans.
	MMP-17	Homology tostromelysin-2	65* 63 A	Pro-MMP2, fibrin/fibrinogen, gelatin.
Matrisylins	MMP-7	Matrisylin	28* 19 A	Collagens IV, gelatin, fibronectin, laminin, elastin, casein, transferrin.
	MMP-26	Matrisylin-2	29	Collagen IV, fibronectin, fibrinogen, gelatin, pro-MMP9.
MT-MMP (membrane type-MMP)	MMP-14	MT1-MMP	66* 54 A	Collagens (I, II, III), gelatin, fibronectin, laminin, vitronectin, entactin, pro-MMP2.
	MMP-15	MT2-MMP	76	Fibronectin, gelatine, vitronectin, entactin, laminin, pro-MMP-2
	MMP-16	MT3-MMP	65* 63 A	Collagen III, gelatin, casein, fibronectin, pro- MMP-2.
	MMP-17	MT4-MMP	65* 63 A	Pro-MMP2, fibrinogen, gelatin.
	MMP-24	MT5-MMP	73	Fibronectin, pro-MMP2, proteoglycans, gelatin.
	MMP-25	MT6-MMP	62	Pro-MMP2, pro-MMP9, collagen IV, gelatine, fibronectin, Proteinase A.
Other enzymes	MMP-12	Macrophage metalloelastase	54* 45 A	Collagen IV, gelatin, elastin, casein, fibronectin, vitronectin, laminin, entactin, fibrin/fibrinogen.
	MMP-19	RASI I	59	Collagen (I, IV) gelatin, fibronectin, laminin.
	MMP-20	Enamelysin	56	Amelogenin, aggrecan.
	MMP-21		65	
	MMP-22		58* 53 A	
	MMP-23		44	gelatin
	MMP-27		59	
	MMP-28	Epilysin	59	

*- Zymogen molecular weight, A active form molecular weight.

Table 1: Human Matrix Metalloproteinases.

leads to more extensive branching, increased ductal elongation, and increased proliferative index; whereas TIMP-1 upregulation leads to inhibition of ductal elongation [11]. Likewise, TIMP-3 deficient mice show accelerated ductal elongation but normal branching patterns [12]. On the other hand, the reversion of a lactating gland to a virgin-like state during involution requires a protease-dependent stage [13]. It has been reported that overexpression of MMP-3 [14] or upregulation of TIMP-1 [15] or TIMP-3 [16], influence mammary regression. These factors have an effect on several substrates during involution, including components of the ECM, proteins involved in cell-cell, and cell-ECM adhesion. In addition, MMPs and TIMPs are implicated in regulating adipogenesis during the late phase of mammary gland involution [17]. Thus, it has been reported that in mouse, MMP-3 contribute to elongate ducts during the mammary gland morphogenesis [18], and its overexpression results in supernumerary ductal branching [19]. Likewise, MMP-2 and MMP-14 deficient mice display diminished ductal elongation, whereas MMP-9 deficiency has no effect [20].

Furthermore, it has been reported that alteration of TIMP levels in mice models also leads to alterations in mammary morphogenesis [12,13, 16]. Also, there is a lot of evidence pointing a key role of the members of the MMP axis in mammary tumorigenesis and in breast cancer progression.

MMPs and TIMPs in Disease and Cancer

Abnormal expression of MMPs contributes to non-neoplastic pathological conditions, involving acute as well as chronic inflammation and/or tissue degradation, and also contributes to cancer [21-25]. There is evidence supporting the hypothesis that inflammation participates in providing conditions that lead to cancer [26,27], and there are well known associations between inflammatory processes and cancer, such as inflammatory bowel disease and colorectal cancer [28,29], viral hepatitis B and C or alcoholic liver cirrhosis and hepatocarcinoma [30], chronic reflux esophagitis resulting in Barrett's esophagus and esophageal carcinoma [29], cervical infection by human papillomavirus

and cervical cancer, prostatitis and prostate cancer, pancreatitis and pancreatic cancer, or gastric infection from *Helicobacter pylori* and gastric cancer [31,32].

MMPs play an essential role in the degradation of the stromal connective tissue and basement membrane components, which are key elements in tumor invasion and metastasis. Thus, it is well established the implication of MMPs in cancer development [33], such as breast cancer [34], colorectal cancer [35,36], prostate cancer [37] and hepatocellular carcinoma [38] among others.

MMP overexpression enhances significantly the invasive and metastatic potential of tumor cell lines both in *in vitro* and *in vivo* studies [39,40]. Genetic mouse models have shown MMPs/TIMPs as tumor modifiers at different levels ([10] for review). During tumor invasion, MMPs appear to have diverse functions, probably due to their substrate preference. It can be assumed that gelatinases are primarily responsible for the destruction of collagen IV in the Basement Membrane (BM), while the stromelysins degrade non-collagenous proteins, such as laminins or extractin/nidogen. After the tumor cells have destroyed the BM and gained contact with the interstitial matrix, the collagenases are required to disrupt the native interstitial collagen network, which is mainly made up by collagen types I, III and V and the microfibrillar collagen IV. In addition, there are data which clearly challenge the classic dogma stating that MMPs promote metastasis solely by modulating the remodeling of ECM, and regarding this, it has been described the MMPs influence tumor cell behavior *in vivo* as a consequence of their ability to cleave growth factors, cell surface receptors, cell adhesion molecules, or chemokines/cytokines [41-44]. Furthermore, by cleaving pro-apoptotic factors, MMPs may induce a more aggressive phenotype via generation of apoptotic resistant cells [45]. MMPs may also regulate tumor angiogenesis, both positively through their ability to mobilize or activate pro-angiogenic factors [46], or negatively via generation of angiogenesis inhibitors, such as angiostatin, endostatin and tumstatin, cleaved from large protein precursors [47].

Nevertheless, in addition to their potential role for inhibiting angiogenesis, there are several studies showing that MMPs can limit tumor progression. For example, in breast cancer, MMP-3 expression in mammary gland decreases mammary tumor development in transgenic mice [48]; also, an increased expression of MMP-8 decreases metastasis of MDA-MB-435 carcinoma cell line both *in vitro* and *in vivo* [49].

MMPs functions seem to depend on their cellular localization. MMPs bound to cell membranes may regulate their activity, leading to the promotion of cell migration and invasion and may activate intracellular signalling cascades [50]. In addition, MMPs on cell surface can be internalized and either directed to the lysosomes for destruction or be a source of intracellular activity. Several MMPs (including MMP-2, -3, -13 and MT1-MMP/MMP-14) have been found in nuclei of various cell types [51-54]. Nuclear localization of MMPs suggested that they may participate in many physiological and pathological cellular processes, in which they can act as both constitutive, regulatory and inducible proteinases [55]. In breast cancer tissue, MMP-1 showed a predominant nuclear immunostaining and a slight cytoplasm staining of tumor cells, whereas normal breast tissue shown no staining for MMP-1 [56]. Also, a nuclear MMP-2 staining was showed in tumor cells; whereas MMP-2 staining was shown in cytoplasm of normal breast endothelial cells [57]. The role of intracellular located MMPs is still poorly understood, and no mechanisms or functions were suggested about the role of nuclear MMPs in the breast cancer processes (for review: [55,58]).

MMPs and TIMPs Expression in Primary Breast Tumors

Several MMPs, specially gelatinases MMP-2 [59-64] and MMP-9 [34,63,65], have been studied as prognostic factors in breast cancer, being associated with poor outcome in various subsets of patients (Table 2). These findings may be due to both MMPs are related to tumor invasion and metastasis by their special capacity to degrade the type IV collagen found in BM [66], and to induce angiogenesis [42]. Likewise, other MMPs or TIMPs may be overexpressed and/or related to clinical outcome in breast cancer, such as MMP-7 [34], MMP-11 [34,60], MT1-MMP (MMP-14) [34,59,67], MMP-13 [68], TIMP-1 [34,69-73] or TIMP-2 [34,73-75], and also the genetic polymorphisms of these proteins may have an association with breast cancer risk, progression and survival [76]. Discordant data about the prognostic value of the above-mentioned MMPs have also been published, and in this way have been related to only a few prognostic factors [77,78] or shown to have no association with clinicopathological parameters in breast cancer [59,79,80].

Histological subtypes of breast carcinoma

It is remarkable that, except for MMP-2, there are significant differences in MMPs and TIMPs expression between the histological subtypes of breast carcinomas [81]. These histological types can be divided into three groups according to the prognostic value: excellent, poor and very poor prognosis. Patients with an excellent prognosis, such as invasive tubular and mucinous carcinoma patients, showed a higher survival rate (over 80%) at 10 years [82], patients with invasive papillary or medullary cancers have a worse prognosis (60-80% survival), and patients with invasive ductal carcinoma and lobular carcinoma were associated with a 10-year survival below 50% [83]. However, only a few studies have evaluated differences in clinical, pathological and biological characteristics according to the histological type. With the increasing incidence of breast carcinoma, the number of patients with an uncommon tumor may increase, so that a more profound knowledge of the molecular biology of these tumors could help to improve the treatment approach. As reported by del Casar *et al.* [81], ductal breast carcinomas showed higher global expression of MMPs and TIMPs than the other histological types; in contrast, mucinous carcinomas had lower expression scores than other carcinomas. With regard to the expression of MMPs and TIMPs in fibroblasts, it was found that these stromal cells were more frequently positive for MMP-1, 7 and 13, and TIMP-1 and 3, in ductal carcinomas than in other histological types of breast carcinomas. With regard to the expression of MMPs and TIMPs in Mononuclear Inflammatory Cells (MICs), these stromal cells were more frequently positive for MMP-1 and TIMP-3, but more often negative for MMP-7, 9 and 11, when located in ductal carcinomas than in other histological types of breast carcinomas. Therefore, variations in MMP/TIMP expression among the histological subtypes of breast carcinomas seems to contribute to the differences in the morphological appearance of breast carcinomas, and might also be related with variations in the tumor pathophysiology of these breast cancer subtypes.

Roles of MMPs and TIMPs in Transition from Ductal Carcinoma *in situ* (DCIS) to Invasive Ductal Carcinoma (IDC) of the Breast

With the adoption of screening mammography, the incidence of Ductal In Situ Carcinoma (DCIS) has risen dramatically, and now DCIS accounts for about 25% of new breast carcinomas cases annually [84].

MMP / TIMP	Expression level	Cancer subtype	Cell type	Correlation with prognosis	Reference	
MMP2	High	-	-	Poor outcome	[60]	
	Positive	T1-2 tumor	-	Shorter survival	[61]	
	High	Lymph node-negative breast cancer	-	Shorter relapse-free survival	[63]	
	High	-	-	Shorter survival	[64]	
MMP9	High	Lymph node-negative breast cancer	-	Shorter relapse-free survival	[63]	
	High	-	Cancer cells	Longer relapse-free survival	[65]	
	Positive	Estrogen receptor positive	Stroma	Shorter relapse-free survival and breast cancer-related survival		
	Low	Invasive ductal carcinoma	-	Shorter relapse-free survival	[34]	
	Positive	Invasive ductal carcinoma	Cancer cells	Shorter relapse-free survival		
	Positive	Invasive ductal carcinoma	Stroma (Fibroblast and MIC)	Shorter relapse-free survival		
	High	Basal like	-	Shorter relapse-free survival		
	MMP7	Positive	Invasive ductal carcinoma	Stroma (Fibroblast and MIC)	Shorter relapse-free survival	[34]
Positive		Invasive ductal carcinoma	Stroma (Fibroblast and MIC)	Shorter relapse-free survival	[34]	
MMP11	High	-	-	Poor outcome	[60]	
	High	Invasive ductal carcinoma	-	Shorter relapse-free survival	[34]	
	Positive	Invasive ductal carcinoma	Stroma (Fibroblast and MIC)	Shorter relapse-free survival		
	High	Basal like	-	Shorter relapse-free survival	[37]	
	Positive	Basal like	Stroma (Fibroblast and MIC)	Shorter relapse-free survival		
	Positive	Luminal A	MIC	Shorter relapse-free survival		
	MMP13	High	Invasive breast carcinomas	Cancer cells	Worse disease outcome	[71]
Positive		Invasive breast carcinomas	Fibroblasts	Adverse overall survival		
MMP14	Positive	Basal like	MIC	Shorter relapse-free survival	[99]	
	Positive	Luminal A	Stroma (Fibroblast and MIC)	Shorter relapse-free survival	[99]	
MMP14	Positive	Invasive ductal carcinoma	MIC	Shorter relapse-free survival	[34]	
	Positive	Luminal A	MIC	Shorter relapse-free survival	100	
	High	-	-	Lymph node metastasis and/or lymph vessel invasion	[67]	
TIMP1	High	Invasive ductal carcinoma	-	Shorter relapse-free survival	[34]	
	Positive	Invasive ductal carcinoma	MIC	Shorter relapse-free survival		
TIMP2	High	Invasive ductal or lobular carcinomas or both	-	Shorter relapse-free survival and shorter survival	[73]	
	High	-	-	Shorter relapse-free survival	[72]	
	High	-	-	Shorter relapse-free survival	[71]	
	High	-	-	Shorter relapse-free survival and shorter overall survival	[70]	
	High	-	-	Shorter relapse-free survival and shorter overall survival	[69]	
	High	Invasive ductal carcinoma	-	Shorter relapse-free survival	[34]	
	Positive	Invasive ductal carcinoma	Cancer cells	Shorter relapse-free survival		
	Positive	Invasive ductal carcinoma	Stroma (Fibroblast and MIC)	Shorter relapse-free survival		
	Positive	Basal like	Stroma (Fibroblast and MIC)	Shorter relapse-free survival		
	TIMP2	Positive	Luminal A	Stroma (Fibroblast and MIC)	Shorter relapse-free survival	[100]
		High	Invasive ductal or lobular carcinomas or both	-	Shorter relapse-free survival	[73]
		High	-	Stroma	Shorter relapse-free survival	[75]
		High	-	-	Shorter relapse-free survival and shorter overall survival	[74]

Table 2: Expression of MMPs and TIMPs and its relationship with clinical outcome of patients with breast cancer.

DCIS of the breast represents a neoplastic proliferation of epithelial cells that is confined to the ductal system and has not extended through the basement membrane. Therefore, in principle, it has no metastatic potential but nevertheless, about 16-22% of DCIS cases develop recurrence after breast-conserving surgery, and about half of them recur as invasive carcinoma, which is more difficult to cure [85]. Even with radiotherapy, there is a 7-9% recurrence rate [86,87], and about half of them are invasive carcinomas [87,88]. Still, little is known about the tumor biology of these pre-invasive cancers.

The transition from DCIS to Invasive Ductal Carcinoma (IDC) of the breast is a poorly understood key event in breast cancer progression [89,90]. Currently, conventional histopathological parameters as those in the Van Nuys classification system are used to identify group of patients with DCIS at high risk to develop recurrence. It has been reported that several markers such as Estrogen Receptors (ER), Progesterone Receptors (PgR), Human Epidermal Growth Factor Receptor 2 (HER2/neu), Ki67, p53, and Bcl-2 correlate with tumor grade and between them, but it is not clear if they are independent

prognostic factors [91]. Performing molecular profiling studies comparing DCIS and IDC could be a great approach to assess cancer progress, although several studies failed to identify tumor-stage-specific signatures [92-95]. Therefore, the analysis of the MMPs/TIMPs expression profile might be an independent factor involved in one or more stages necessary for progression from pre-invasive to invasive tumors and, consequently, it may have a role in predicting recurrence in DCIS.

Previous reports showed the expression of MMP-1, 2 [96,97], 3, 9 [96], and TIMP-1 [97] in the stroma around non-invasive breast tumors, although other reports showed no significant differences in the expression levels of some MMPs between DCIS and IDC [98]. It is of mention the possible relationship between the MMP/TIMP expressions in both DCIS and IDC components of mixed cases. Only a significant but poor correlation for MMP-2 score values between both lesions has been found. However, significant differences between both paired set of lesions for MMP-1, 2, 7, 9 13, 14, and TIMP-1 expression by tumor cells were found [99-100]. It suggests that the step from DCIS to an invasive phenotype implicates some significant changes in the MMP/TIMP expression profile, either by the intraductal tumor cells in the neoplastic ducts, or by the stromal cells between them.

Recently, we found variability in MMP/TIMP expression in pure DCIS, with a group of these lesions showing higher expression of MMP-1, 9, 11, 13, 14, TIMP-1, 2, and 3 [100], which may be of potential biological and clinical interest to predict DCIS evolutionary behavior towards IDC. Further and prospective studies are necessary to confirm the clinical value of this molecular profile to predict the evolutionary behavior towards IDC. Nevertheless, it seems interesting to compare MMP/TIMP expressions between different lesions, such as pure DCIS, IDC, DCIS mixed with IDC, both in DCIS and invasive components in mixed cases of breast cancer, or with focus in the microinvasive events in DCIS (Figure 3).

When we compare MMPs/TIMPs expressions, it was remarkable that MMP-7 and TIMP-1 expressions were significantly higher in IDC than in pure DCIS [100]. It has been reported that MMP-7 overexpression enhances cellular invasiveness and activation of proMMP-2 and MMP-9 [101], which supports the role of MMP-7 in tumor invasion in breast cancer. With regard to TIMP-1, it may have tumor-stimulatory functions, such as promote cell proliferation [102].

Analysis of pure DCIS showed higher levels of MMP-9 [100,103] MMP-11 and TIMP-3 [100] than the DCIS component of mixed cases. This suggests that, MMP-9 may play a role in the very early stages of DCIS development, but once the BM has been broken, cancer cells become less dependent on its activity [103]. TIMP-3 may be relevant considering the putative role in tumor growth inhibition, as it antagonizes primary tumor growth, angiogenesis, apoptosis, tumor invasion, and the development of metastasis [104-108]. Recent studies about the methylation-associated silencing of TIMP-3 also suggest a tumor suppressor role in several tumor types [109-113], which might not be directly related to its effect as an MMP inhibitor. Therefore, the lack of TIMP-3 expression in pure DCIS may be a potential marker of invasive growth. It also was of note the existence of a positive and significant association between the peri-ductal inflammatory infiltrate and MMP-2, 14, or TIMP-1 scores in pure DCIS [100]. These associations are interesting considering that these factors may stimulate peri-ductal angiogenesis, and therefore B and T-cells may be recruited via high endothelial venules induced by DCIS. These dates indicates that the inflammatory infiltrate could also affect angiogenesis by releasing angiogenic factors [114].

In contrast, MMP-1 expression was significantly higher in DCIS than in the corresponding IDC in mixed cases [99]. MMP-1 is required for local invasion due to its ability to degrade the type I collagen (the principal component of connective tissue) [4]. High expression of MMP-1 by fibroblast cells correlated with the occurrence of distant metastasis, which is in accordance with previous studies showing its association with an elevated metastasis capacity [115]. In addition, MMP-1 expression by mononuclear cells is associated with sequential metastasis across lymph nodes in breast cancer [116]. Thus, the lower expression of MMP-1 in the IDC component may suggest a less aggressive evolutionary development of invasiveness in these carcinomas with mixed components. Accordingly with this concept, it has been shown that pure IDC showed significant higher expression of MMP-1, 9, 11 14, TIMP-1 and 3, than the IDC component of mixed cases, suggesting a more aggressive behavior of pure IDC. In this line, several clinical studies have shown that the presence of a prior, simultaneous, or subsequent breast carcinoma *in situ* is associated with a better survival for patients with IDC [117]. This improved survival may have an immunological basis. Nevertheless, if we assume that DCIS preceded the elements showing stromal infiltration, we can also consider that pure IDC may be characterized by a fast growth which quickly obliterate and/or destroy the neoplastic ducts of the precursor lesions, a hypothesis supported by the higher MMP/TIMP molecular profile in pure IDC.

Our Group found no significant differences in MMPs/TIMPs expression between intraductal tumor cells and tumor cells from microinvasive foci, both belonging to DCIS with microinvasion [118]. However, there was a significantly higher MMP-13 expression in fibroblasts and MMP-14 expression in MICs from invasive foci, compared with the respective paired expression in peri-ductal fibroblasts or in peri-ductal MICs from the neoplastic ducts. These data are in accordance with a previous study by Nielsen et al. [68], who reported that MMP-13 expression by myofibroblasts was often associated with microinvasive events and, thus this collagenase could play an essential role during the transition from DCIS to IDC of the breast. As mentioned above, MMP-13 and MMP-14 have an exceptionally and wide role in molecular carcinogenesis, tumor cell growth, invasion and angiogenesis. Over the past few years, accumulated evidences indicate that both changes in stromal behavior and tumor/stroma cell interactions are intimately linked to the processes of tumorigenesis, tumor invasion, and metastasis [119]. Thus, all these data suggest a relevant role of peri-ductal stromal cells in the early phases of tumor invasion in breast cancer.

On the basis of all of these data, variations in the MMP/TIMP expression, either by tumor cells or by stromal cells, seem to have an essential role in the potential invasive phenotype in DCIS. Thus, analysis of the MMP/TIMP molecular profile can contribute to a better characterization of early breast carcinomas which differ in their biological and clinical behavior. Therefore, the staining patterns of MMP/TIMP might display potential applications as biological markers, such as in the evaluation of microinvasion in resection specimens of breast tumors. Nevertheless, it is necessary to study a large number of DCIS cases with long follow-up focused on invasive recurrence, to evaluate the predictive and prognostic value of MMPs/TIMPs expression. Since the identification of a molecular profile associated with tumor recurrence after breast conservative surgery, is highly desirable to recognize the majority of DCIS patients with a very low risk of developing invasive recurrence, which will not need radiotherapy after breast conservative surgery, to avoid overtreatment and side effects in these patients

MMPs and TIMPs in Stroma

Breast cancer, as a solid tumor, consists of a variable mixture of neoplastic cells and non-neoplastic tumor stroma cells, comprising endothelial cells, pericytes, fibroblast and a variable representation of inflammatory cells. Over the past few years, several evidences have shown that both changes in the stromal behavior and the interaction between tumor cells and stromal cells, are intimately linked to tumorigenesis, tumor invasion and metastasis [119,120]. In fact, it is currently known that in addition to their production by epithelial tumor cells, MMPs and/or TIMPs expression may be induced in infiltrating stromal fibroblasts and/or in vascular and inflammatory cells [68,71,75,121]. Therefore, the main source of MMPs in breast carcinoma are the stromal cells [122-125], and also experimental studies have demonstrated that the mechanism by which breast cancer cells can rapidly use MMPs produced by adjacent normal fibroblasts to facilitate their invasion into the peritumoral tissue [126].

Nowadays, it is widely accepted that the cellular type (tumor cell/stromal cell) expressing these individuals factors might have a biological interest in breast cancer. Thus, we found that the expression of MMP-9 or TIMP-2 by tumor cells, MMP-1, 7, 9, 11, 13, or TIMP-3 by fibroblasts, and MMP-7, 9, 11, 13, 14, or TIMP-1 and 2 by MICs, was significantly associated with a higher rate of distant metastases [34].

MMPs and TIMPs expression in MICs

Inflammatory cells can account for as much as 50% of the total tumor mass in invasive breast carcinomas. MICs infiltrate in breast carcinomas include a variable representation of macrophages, plasma cells, mast cells and B and T-lymphocytes [127,128]. Historically, tumor-infiltrating leukocytes have been considered to be manifestations of an intrinsic defense mechanism against developing tumors [128,129]. However, our data are in accordance with the increasing evidences indicating that leukocyte infiltration can promote tumor phenotypes, such as angiogenesis, growth and invasion [127,130]. This may be due to inflammatory cells, which secrete cytokines, growth factors, chemokines and proteases, stimulating cancer cell proliferation and invasiveness [131]. Nevertheless, the prognostic significance of the lymphoid infiltrate at the tumor site remains controversial, perhaps because the evaluation criteria for tumor infiltrates are not sufficiently standardized to yield reliable and reproducible results in different institutions.

An unsupervised hierarchical cluster analysis identified two cluster groups, one consisting of tumors showing a low, and another showing a high MMP/TIMP expression profile by intratumoral MICs [34], and this latter strongly associated with distant metastasis development (Figure 1). Thereby, multivariate analysis indicates that to belong to this cluster group is the most significant and independent prognostic factor to predict distant metastasis development in patients with IDC [132]. In addition, these two differential MICs phenotypes with distinct prognosis were also found in breast carcinomas with luminal A or in basal-like phenotype [133], which suggests the importance of the expression of MMPs/TIMPs by the stromal cells as prognostic factors independently of the signature of cancer cells. These two tumor groups were present at the invasive front of tumors, but it was also possible to identify a third group of tumors who's MICs showed an intermediate MMP/TIMP expression profile [134]. These findings suggest that tumor-infiltrating leukocytes from peripheral blood undergo a phenotypic modification to infiltrate from the invasive front into the tumor center. This seems to be a dynamic process in which inflammatory cells and immunomodulatory mediators present in the

tumor microenvironment polarize the host immune response towards specific phenotypes impacting on tumor progression. Patients with high MMP/TIMP expression patterns in the corresponding MICs populations at the tumor center, as well as at the invasive front, had the highest probability to develop distant metastases, indicating the importance of evaluating the expression of these factors involved in tumor growth by MICs located in different tumor areas, which provide complementary information about tumor behavior and prognosis in breast cancer.

It is interesting to describe some biological characteristics of the MMPs expressed by this prometastatic-related MICs, specifically MMP-7, 9, 11, 13 and 14, and TIMP-1 and 2 [132]. MMP-7 (matrilysin 1) is a stromelysin which degrades type IV collagen, fibronectin and laminin, that is aberrantly expressed in human breast tumors, and whose elimination is associated with lower invasiveness and reduced tumor growth [135]. MMP-9 (gelatinase B) is related to tumor invasion and metastasis by their special capacity to degrade the type IV collagen found in BM [66], and is also able to induce angiogenesis [42]. Indeed, a high MMP-9 expression correlates significantly with tumor aggressiveness and poor prognosis [63, 65]. MMP-11 (Stromalysin-3) was preferentially expressed by peritumoral stromal cells [136,137], and high levels of MMP-11 were associated with tumor progression and poor prognosis [34,71]. MMP-13 (collagenase-3) has an exceptionally wide substrate specificity when compared with other MMPs [138,139], play a central role in the MMP activation cascade, both activating and being activated by other MMPs (MMP-14, 2 or 3), and may play an essential role during the transition from DCIS lesions to IDC of the breast [68]. MMP-14 (membrane type 1 MMP or MT1-MMP) is a key MMP involved in the degradation of ECM, activation of pro-MMP-13 [140] and pro-MMP-2 [141] in the cell surface, and plays crucial roles in molecular carcinogenesis, tumor cell growth, invasion and angiogenesis.

The positive relationship between TIMP expression by MICs and cancer progression may appear paradoxical, because both TIMP-1 and 2 are well-known inhibitors of MMP activity. As TIMPs inhibit MMPs *in vivo*, it should be expected that high levels of inhibitors would

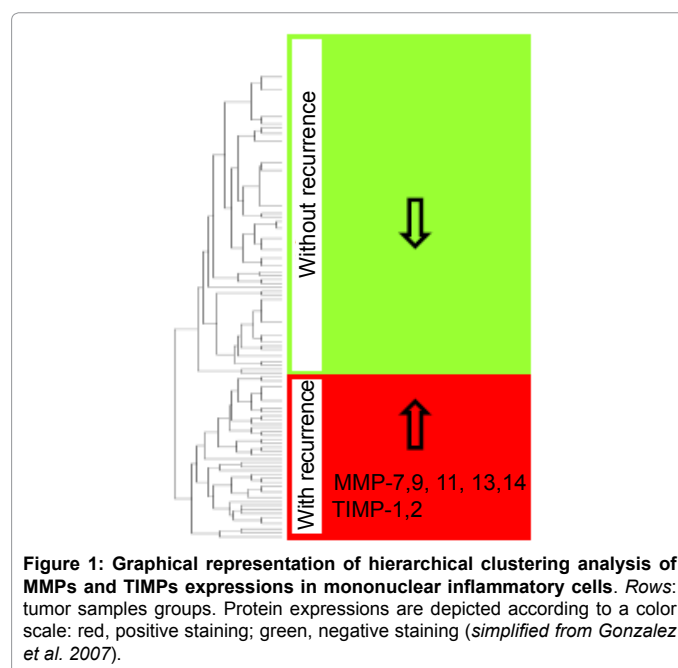


Figure 1: Graphical representation of hierarchical clustering analysis of MMPs and TIMPs expressions in mononuclear inflammatory cells. Rows: tumor samples groups. Protein expressions are depicted according to a color scale: red, positive staining; green, negative staining (simplified from Gonzalez et al. 2007).

prevent tumor progression and thus should be related to good outcome in cancer patients. However, they may also promote cell proliferation and have antiapoptotic effects that may favor tumor expansion during the onset and early growth of primary tumors [102,107,142,143].

MMPs and TIMPs expression in fibroblasts

Fibroblast is one of the main stromal cellular components of breast carcinomas. Clustering analysis showed two distinct groups, with low or high MMP/TIMP molecular profiles in both fibroblast populations, either in the tumor center or in the invasive front, but each of them with different MMP/TIMP patterns. Intratumoral fibroblasts showed a positive expression of MMP-2, 7 and 14, and TIMP-3 more frequently than fibroblasts at the invasive front, which showed a more frequently expression of MMP-9 (Figure 2). This varied expression pattern of MMPs and TIMPs may correspond to differences in cellular density, which is higher in the tumor center, and/or to biological mechanisms of interaction between tumor cells and the fibroblast population of those two different tumor areas [144]. Accordingly, it has been shown that cell-cell contact between cancer cells and fibroblasts enhanced the production and activation of MMPs by cancer cells, promoting pericellular proteolysis, angiogenesis and tumor cell invasion [145,146]. The expression of MMPs and TIMPs by fibroblasts is an independent factor predicting the occurrence of distant metastases, depending on the tumor location of those cells. Thus, whereas in fibroblasts at the tumor center the expression of MMP-9, 13 and TIMP-3 was associated with distant metastases, in the fibroblasts at the invasive front was the expression of MMP-14 and TIMP-1. However, patients with high MMP/TIMP patterns in the fibroblast population at the tumor center as well as at the invasive front had the highest probability of distant metastases, whereas patients with low MMP/TIMP patterns in both fibroblast populations had the lowest risk of distant metastases [144].

All of these findings led us to consider the importance of the expression of MMPs and TIMPs by stromal cells in the different areas of breast carcinomas, in order to assess the clinical relevance of this tumor heterogeneity, as well as to achieve a better knowledge about the role of stromal cells in breast cancer progression. All of these show the importance of the largely unknown contribution of the tumor environment to the malignant phenotype. Historically, the importance of tumor microenvironment during cancer progression was recognized more 100 years ago in the “seed and soil” hypothesis proposed by Paget in 1989 [147]. Therefore we can conclude, such as Noël et al. [148], that MMPs seem to be molecular determinants of Paget’s “seed and soil” concept.

MMPs and TIMPs Expression in Metastasis

Role in distant metastasis development

As described more above, patients with high MMP/TIMP expression pattern in the corresponding MICs populations at the tumor center, as well as at the invasive front, had the highest probability to develop distant metastases [134]. Also, we demonstrated that MMP-11 was the most frequently expressed protein in these prometastatic-related MICs (85.7% vs. 4.6% in the low MMPs/TIMPs profile group), and therefore its expression was considered as a useful biological marker in these MICs population [132]. Previously, high levels of MMP-11 had been associated with tumor progression and poor prognosis [71]. On the basis of this finding, and after the analysis carried out by real-time PCR of 65 factors associated with tumor progression and inflammation, Eiró et al. [149,150] recently reported that 22 factors were related with MMP-11 expression by MICs. Of them, factors more

differentially expressed between both groups of tumors were IL-1, 5, 6, 17, IFN β and NF κ B. Altogether, these results indicate that tumors developing worse prognosis and identified by MMP-11 expression by intratumoral MICs, showed an up-regulation of inflammatory-related genes. These associations are relevant because these highly expressed genes have been associated with several biological mechanisms related to tumor progression [151-157]. It is also relevant the novel finding of the association between the expression of MMP-11 or TIMP-2 by the MICs at the tumor center and a high CD68/(CD3+CD20) ratio (macrophages (CD68⁺), T-cells (CD3⁺) and B-cells (CD20⁺)) [158], since both proteins are the two principal factors defining the prometastatic phenotype of MICs in our previous studies [34,132,134,144]. In addition, if there is a high CD68/(CD3+CD20) ratio at the invasive front, most of MICs with a positive MMP-11 or TIMP-2 phenotype at the tumor center are macrophages, suggesting all these findings that a high CD68/(CD3+CD20) ratio at the invasive front contributes to polarize macrophages to achieve a high metastatic phenotype at the tumor center.

Role in lymphatic metastasis

Classically, biological and/or prognostic factors in breast cancer have been investigated in the primary tumor. However, draining lymph nodes, and specially Sentinel Lymph Nodes (SLNs), are of great interest because they are exposed to all soluble factors coming from the tumor and may also be colonized by aggressive clones deriving from primary tumor cells. It has been reported that when comparing MMPs/TIMPs immunostaining values between different tumor localizations (tumor center, invasive front or Metastatic Axillary Lymph Nodes (MALNs)), the higher positive correlations were found between MALNs [159], suggesting that clones of primary tumor cells which colonize regional lymph nodes show a tendency to have a similar phenotype of MMPs/TIMPs.

It was recently shown that specific MMP/TIMP expression by MALNs (such as MMP-1, 7, 13 or TIMP-1 by MICs) was associated with the number of invaded nodes. Likewise, it is especially relevant that MMP-1 (interstitial collagenase, also named collagenase-1) expression by MICs from SLNs was significantly associated with metastatic spread to non-SLNs. This seems to indicate that metastatic cancer cells have the ability to induce the production of these proteins

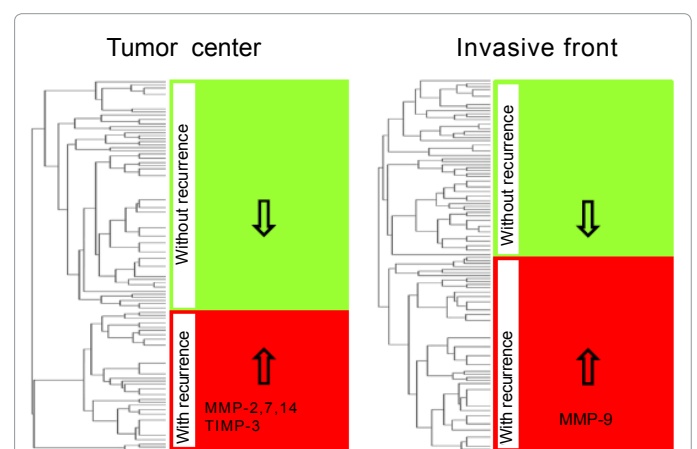
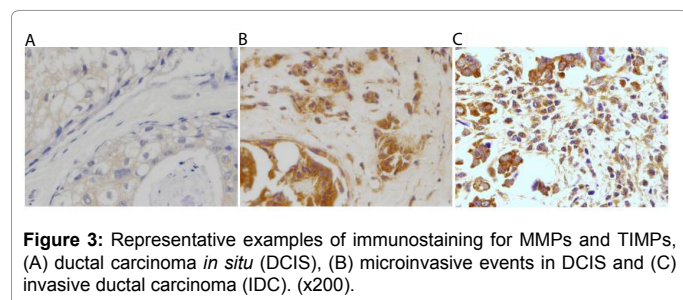


Figure 2: Graphical representation of hierarchical clustering analysis of MMPs and TIMPs expressions in fibroblasts in the center of the tumor, and fibroblasts at the invasive front. Rows: tumor samples groups. Protein expressions are depicted according to a color scale: red, positive staining; green, negative staining (simplified from Del Casar et al. 2009).



in the inflammatory host cells within the lymph nodes, which emphasizes the importance of the stromal-epithelial interactions in the tumor progression among MALNs. In addition, it was reported that in all cases with negative MMP-1 expression by MICs from SLNs, the remaining non-SLNs were not affected, pointing to a 100% sensitivity, a 100% negative-predictive value and a 61.5% specificity to predict non-SLNs status [116]. Therefore, if confirmed in larger studies, MMP-1 expression by MICs from SLNs may be a useful biological marker to predict metastatic progression across the axillary lymphatic system in breast cancer, which could help to avoid unnecessary axillary node dissection in a significant percentage of cases (50-68%) [160,161] when metastatic spread to other axillary nodes, apart from SLN, is suspected.

MMP-1 is the most ubiquitously expressed of the interstitial collagenases. MMP-1 cleaves several components of the extracellular matrix, including collagen type I (the principal component of the connective tissue), II, III, VII, VIII, and IX, aggrecan, as well as serin-protease inhibitors, and $\alpha 2$ macroglobulin [4]. The reported data by Eiró et al. [116] seem to indicate that the degradation capacity of MMP-1 may be responsible for promoting tumor spread via the lymph nodes. This observation may appear in contradiction with those previously reported regarding the lack of association between high MMP-1 expression by MICs from primary tumors and distant metastasis in breast carcinomas [132]. However, the metastatic progression across the axillary lymphatic system is a process completely different from hematogenous tumor spread, which is the one responsible for distant metastases [162,163].

MMPs as Therapeutic Targets

Based on the findings about MMPs overexpression in malignant tumors, diverse synthetic MMP inhibitors (MMPIs) have been developed as potential therapeutic agents against cancer [164,165]. Several generations of synthetic MMPIs have been tested in phase III clinical trials in humans, and include three classes of inhibitors: peptidomimetics, non-peptidomimetics inhibitors and tetracycline derivatives, which target MMPs in the extracellular space. The peptidomimetic MMPIs mimic the collagen structure at the MMP cleavage site, functioning as competitive inhibitors, and chelating the zinc ion present at the activation site [166]. Batimastat (BB-94) and marimastat are hydroxymate-based inhibitors belonging to this MMPIs group, and have been associated with musculoskeletal syndrome, probably as a result of their broad spectrum of inhibition [167-169]. In addition, *in vitro* studies with these MMPIs showed that they can act synergistically with TIMP-2 in the promotion of proMMP-2 activation by MMP-14, increasing the overall pericellular proteolysis [170]. On the other hand, the non-peptidomimetic MMPIs (tanomastat (BAY12-9566), prinomastat (AG3340), BMS-275291 and CGS27023A) have improved specificity and oral bioavailability [171], but musculoskeletal side effects and limited efficacy were also reported in clinical trials [164,172]. The chemically modified tetracyclines derivatives (metastat

(COL-3), minocycline and doxycycline) are MMPIs that inhibit both the enzymatic activity and the synthesis of MMPs via blocking gene transcription. These inhibitors lacking antibiotic activities, may inhibit MMPs by binding to metal ions such as zinc and calcium, and cause limited systemic toxicity compared to regular tetracyclines. Among these MMPIs, doxycycline is currently the only approved by the Food and Drug Administration for periodontitis prevention, whereas metastat has entered in phase II trials for Kaposi's sarcoma and brain tumors treatment [173].

The majority of synthetic MMPIs used in clinical trials of late-stage malignancies showed only a borderline beneficial effect [174,175], and in some cases their activity was associated with a negative patient outcome [176,177]. Administration of many of these broad spectrum MMPIs was also accompanied by dose limiting side effects including muscle and bone pain, maybe due to the complexity of the pro- and anti-tumorigenic roles described for MMPs and TIMPs, since the protective effects of MMPs, especially in processes such angiogenesis, were not available at the time of the first clinical trials [178]. To date, clinical trials with MMPIs have been performed in unselected patient populations, often with late-stage disease, which may be relevant because MMPIs are more effective in early but not late cancer stages [179]. It is also of note that MMPs may also play anti-tumor functions in many tumors, as well. For example, MMP8^{-/-} mice developed more papillomas upon carcinogen treatment [180].

To avoid the negative results and toxicity issues raised by the use of synthetic MMPIs, various natural compounds have been identified as MMPs inhibitors. TIMPs have demonstrated efficacy in experimental models to block MMPs activity, but TIMPs may exert MMP-independent promoting effects [181]. Another natural compounds such as neovastat (extracted from shark cartilage) [182] or genistein (a soy isoflavonoid structurally similar to estradiol) [161,183] have anticancer effects, in part interfering with the activity of several MMPs. There are other drugs that influence MMPs, like bisphosphonates, which inhibit the enzymatic activity of various MMPs [184]. In *in vitro* studies, addition of letrozole, a reversible nonsteroidal inhibitor of P450 aromatase, considerably suppressed the activity of gelatinases (MMP-2 and -9) released by breast cancer cells, as well as invasion, limiting the metastatic potential of these cells [185]. These latter data are in accordance with the results obtained in the British International Group 1-98 study showing that letrozole decreases the occurrence of distant metastases [186]. Curcumin (diferuloylmethane) is a polyphenol derived from the plant turmeric (*Curcuma longa*), commonly used as a spice. It has been show that curcumin inhibits 12-O-Tetradecanoylphorbol-13-Acetate (TPA)-induced MMP-9 expression and cell invasion through suppressing NF- κ B and AP-1 activation in MCF-7 cells [187]. Cysteamine, an anti-oxidant aminothioliol, is the treatment of choice for nephropathic cystinosis, a rare lysosomal storage disease. Similar to the *in vitro* results, MMP activity was significantly decreased in animal cysteamine-treated tumors [188].

There are other strategies to inhibit MMPs activity. Strategies involving antisense and small interfering RNA (siRNA) technology directed selectively against mRNA of a specific MMP, resulting in decrease of RNA translation and down-regulation of MMP synthesis, are in development [135,189]. In addition, efforts have been made towards the development of very specific MMPIs. The fully human monoclonal antibody DX-2400 (Dyax Corp.) that targets MMP-14, has shown great promise in preclinical models in inhibiting invasiveness of cancer cell lines [190]. In addition, a novel class of MMPIs, the triple-helical transition state analogues, specifically targets the gelatinase and

collagenase activities of metalloproteases (specifically MMP-2 and 9) [191].

Addressing clinically the role of MMPs in breast cancer metastasis will involve not only the design of trials to maximize the impact of the therapeutics, but also developing MMPi with a highly selective and specific MMP inhibition, taken care to target only relevant MMPs in each specific disease setting. In addition, greater knowledge is needed concerning stromal *versus* epithelial expression of MMPs [192].

Conclusions

MMPs and TIMPs play a key role in several basic processes of tumor progression. Different expression profiles are associated with the main steps of breast cancer progression, such as creating a potential invasive phenotype in DCIS, favoring the hematogenous development, and making possible the metastatic progression across the axillary lymphatic system. These associations have clinical interests, as they can contribute to a better characterization of early breast carcinomas which differ in their biological and clinical behavior, to evaluate microinvasion in resection specimens of breast tumors, to provide a more precise prognostic, and for predicting the tumor status of non-SLNs in breast cancer. It is also especially remarkable the evidences indicating that MMPs and TIMPs expression in individual cell populations in the tumor stroma, such as MICs and fibroblasts, clearly impact on clinical outcomes in breast cancer patients, suggesting that tumor stroma not only does not merely play a passive role in cancer progression, but also may actively participate in the cancer invasion process. Likewise, analysis of the expression profiles revealed clinically relevant correlations between individual MMP expression and immune cell involvement in breast cancer. Since there are several factors linking inflammation, MMP activity, and breast cancer, all this knowledge will serve to drive novel therapies and prevention strategies targeting critical components [10]. Thus, for example, the finding of a MICs phenotype infiltrating tumors, characterized by the expression of a specific panel of MMPs and TIMPs, strongly associated with the development of distant metastasis, suggests that these host inflammatory cells could be a possible target for the inhibition of tumor progression and metastasis.

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